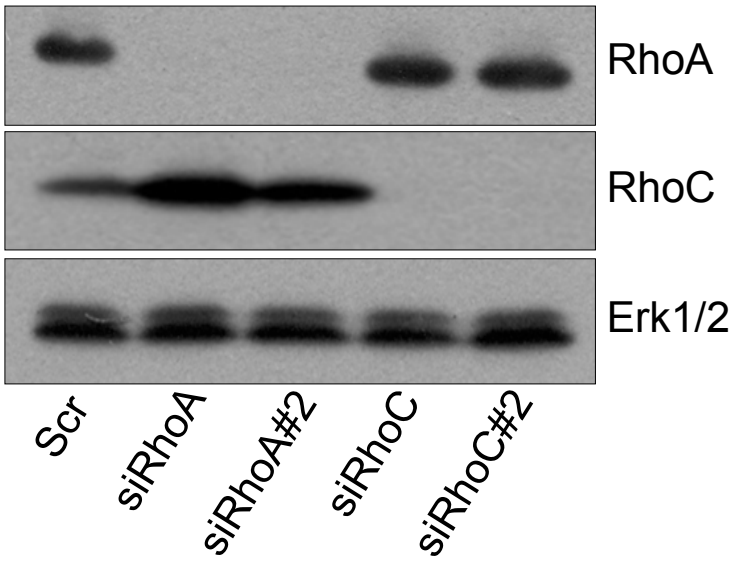
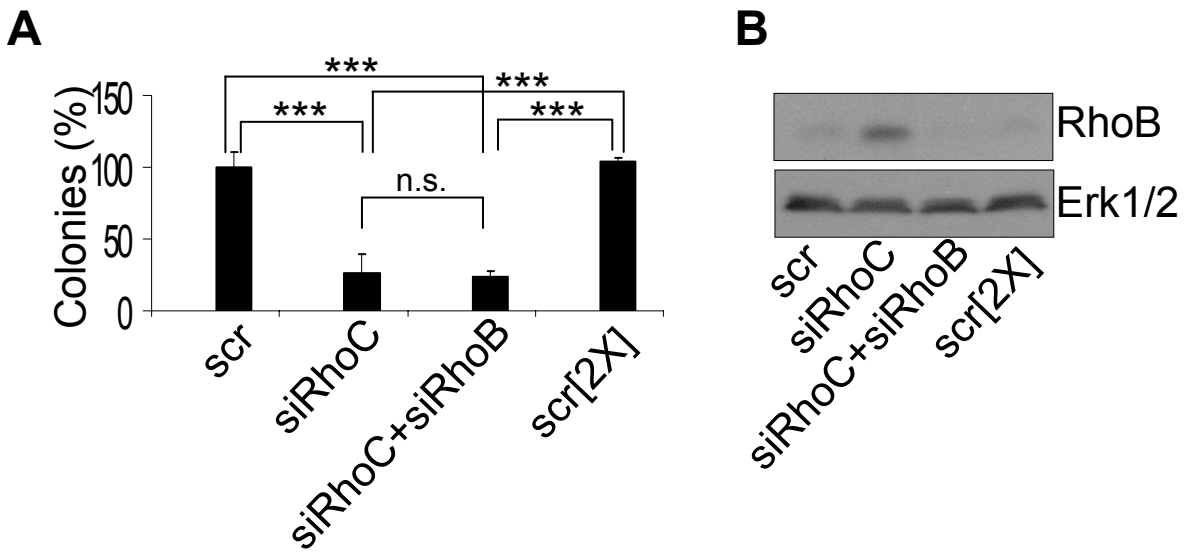
Supplemental Figure 1



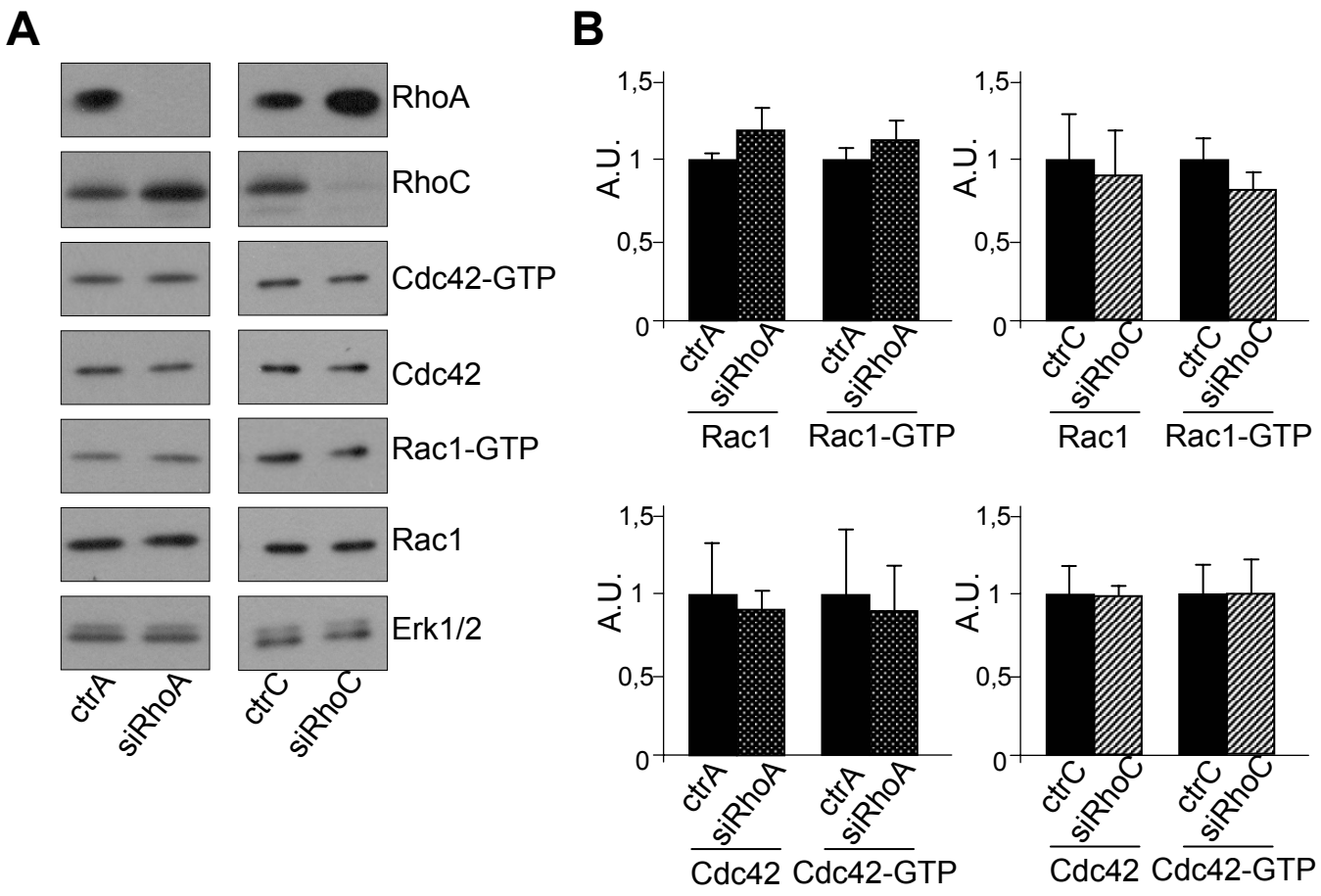
RhoA and RhoC were efficiently silenced with a second siRNA.
PC-3 cells were transfected by 20nM of two different siRNAs targeting RhoA (siRhoA and siRhoA#2), by 20nM of two different siRNAs targeting RhoC (siRhoC and siRhoC#2) and by an irrelevant siRNA as control (Scr). 48 hours post transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to RhoA, RhoC and Erk 1/2. Representative blots of three independent experiments are shown.

Supplemental Figure 2



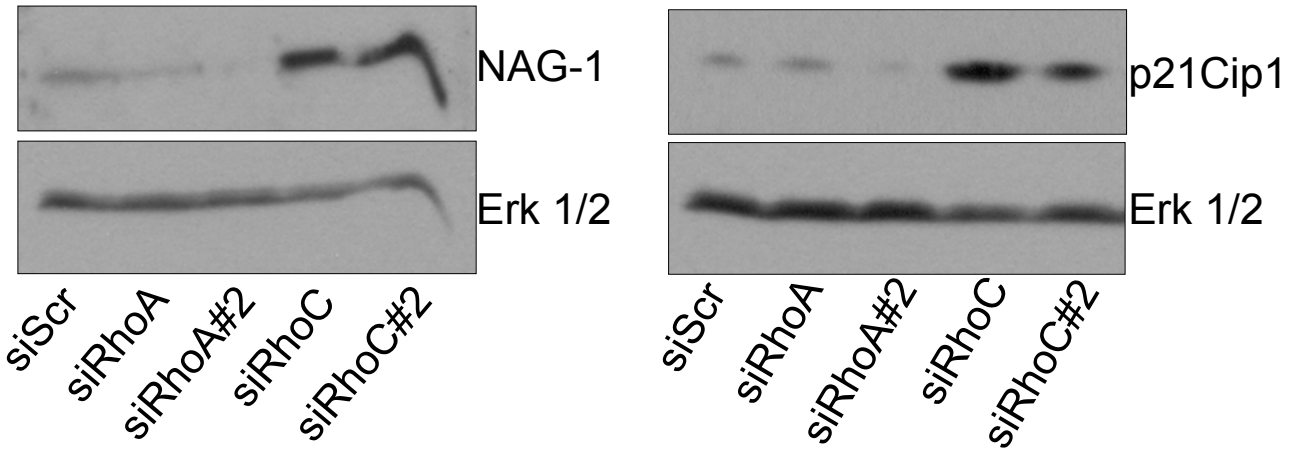
The overexpression of RhoB following RhoC silencing is not involved in the inhibition of growth in soft agar. (A) $2x10^3$ PC-3 cells transfected with 20 or 40nM of an irrrelevant siRNA (scr or scr[2X]), with 20nM of siRhoC (siRhoC) or with 20nM of siRhoC + 20nM of siRhoB (siRhoC+siRhoB) were plated as described in Materials and Methods. After 15 days of culture, colonies were counted in the whole dishes. Results are reported as percentages of values obtained with the irrelevant siRNA (scr) and are mean±s.d. of three independent experiments. n.s. not significant and *** $p< 0.001$ ANOVA followed by Tukey-Kramer analysis. Results are representative of two independent experiments. (B) Western blot analysis of lysates from PC-3 cells 48 hours after transfection with the indicated siRNA.

Supplemental Figure 3



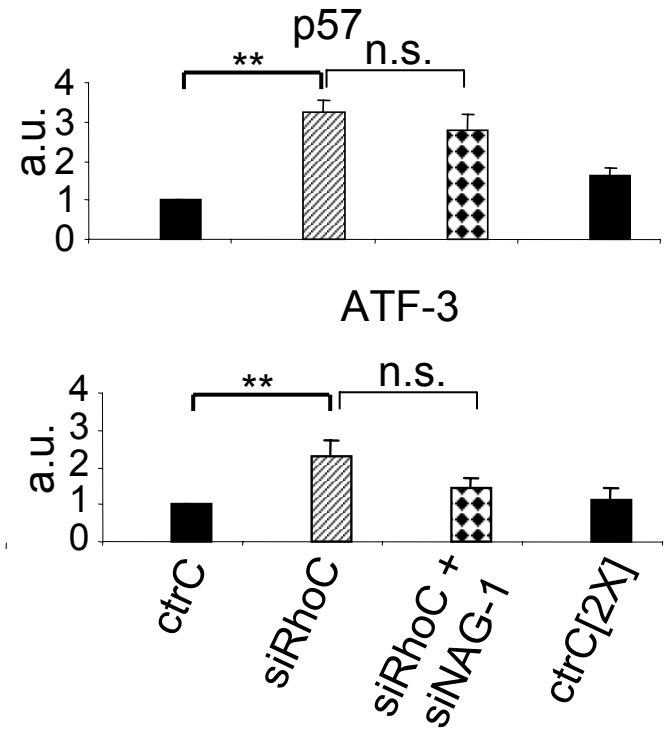
Neither RhoA-silencing nor RhoC-silencing affected the Rac1 or Cdc42 expression level and activity. PC-3 cells were transfected with 20 nM of the indicated siRNA. 48h after transfection, cells were harvested and processed for Western blot and pull-down experiments. An aliquot of each lysate was denatured in SDS-PAGE loading buffer to analyse the concentration of total Rac1, Cdc42, RhoA, RhoC and Erk1/2 with specific antibodies. Erk1/2 was used as loading control. Rac1 and Cdc42 activities were determined as the amount of GST-PBD-bound Rac1 (Rac1-GTP) or GST-PBD-bound Cdc42 (Cdc42-GTP) normalized respectively to total Rac1 or total Cdc42 in whole cell lysates. In A, representative blots are shown. The panels in B illustrate the mean±s.d. of 3 independent experiments. Results are expressed in arbitrary units (A.U.).

Supplemental Figure 4



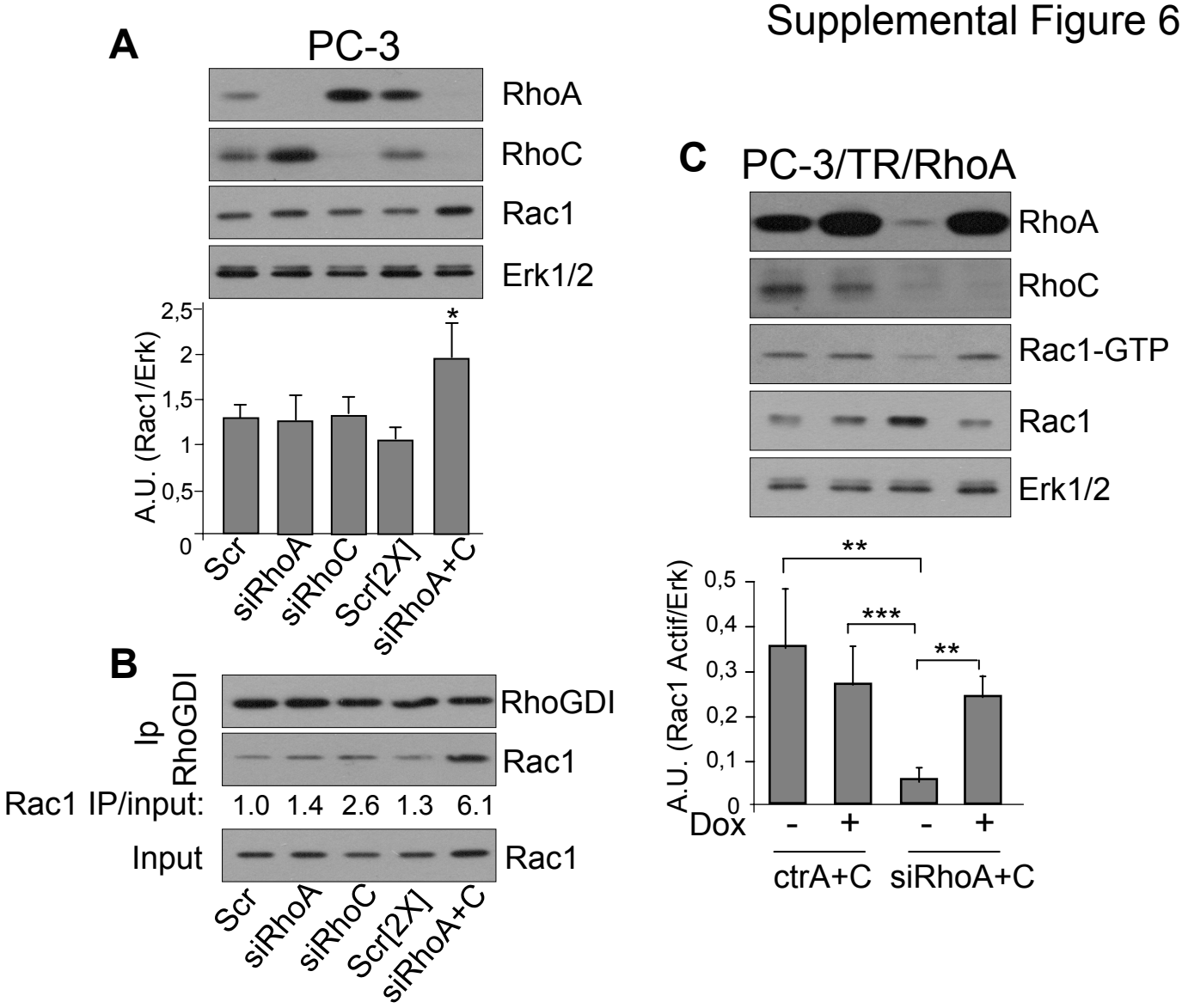
The up-regulation of NAG-1 and p21Cip1 protein level following RhoC-silencing were confirmed with a second siRNA targeting RhoC (siRhoC#2).
PC-3 cells were transfected by 20nM of two different siRNAs targeting RhoA (siRhoA and siRhoA#2), by 20nM of two different siRNAs targeting RhoC (siRhoC and siRhoC#2) and by an irrelevant siRNA as control (siScr). 48 hours post transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to NAG-1, p21Cip1 and Erk 1/2. Representative blots of three independent experiments are shown.

Supplemental Figure 5



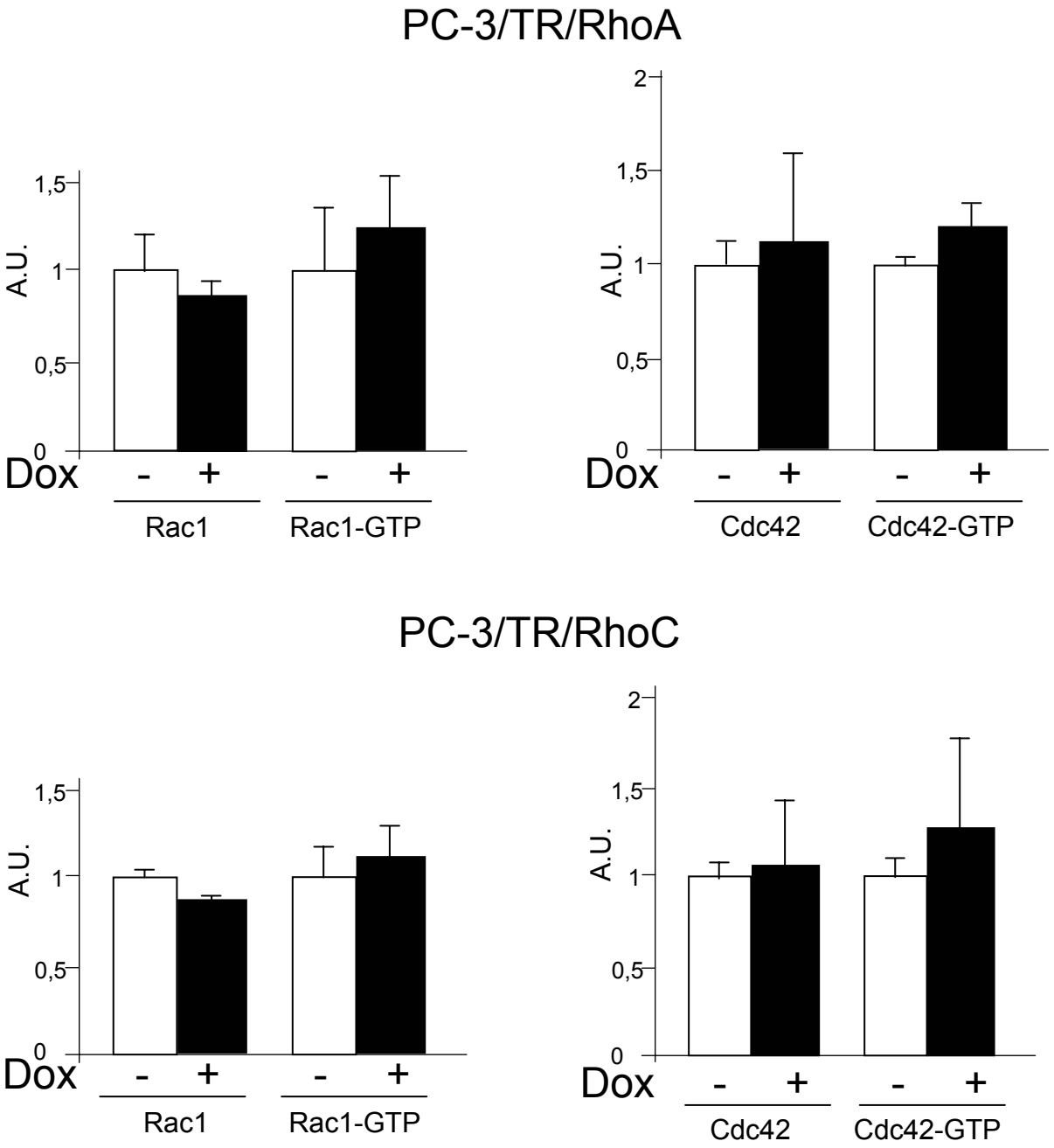
The expression of p57 and ATF-3 are independent of NAG-1.
Real time quantitative PCR analysis of the expression of the indicated mRNA was performed with total RNA extracted from PC-3 cells 48 hours after transfection with 20nM (or 40nM) of specific control for siRhoC (ctrC or ctrC[2X]), with 20nM of siRhoC or with 20nM of siRhoC + 20nM of siNAG-1.
Results expressed in arbitrary units (a.u.) relative to ctrC taken as 1 are the mean±sd of three independent experiments. **p<0.01 ANOVA followed by Tukey-Kramer analysis.

Supplemental Figure 6



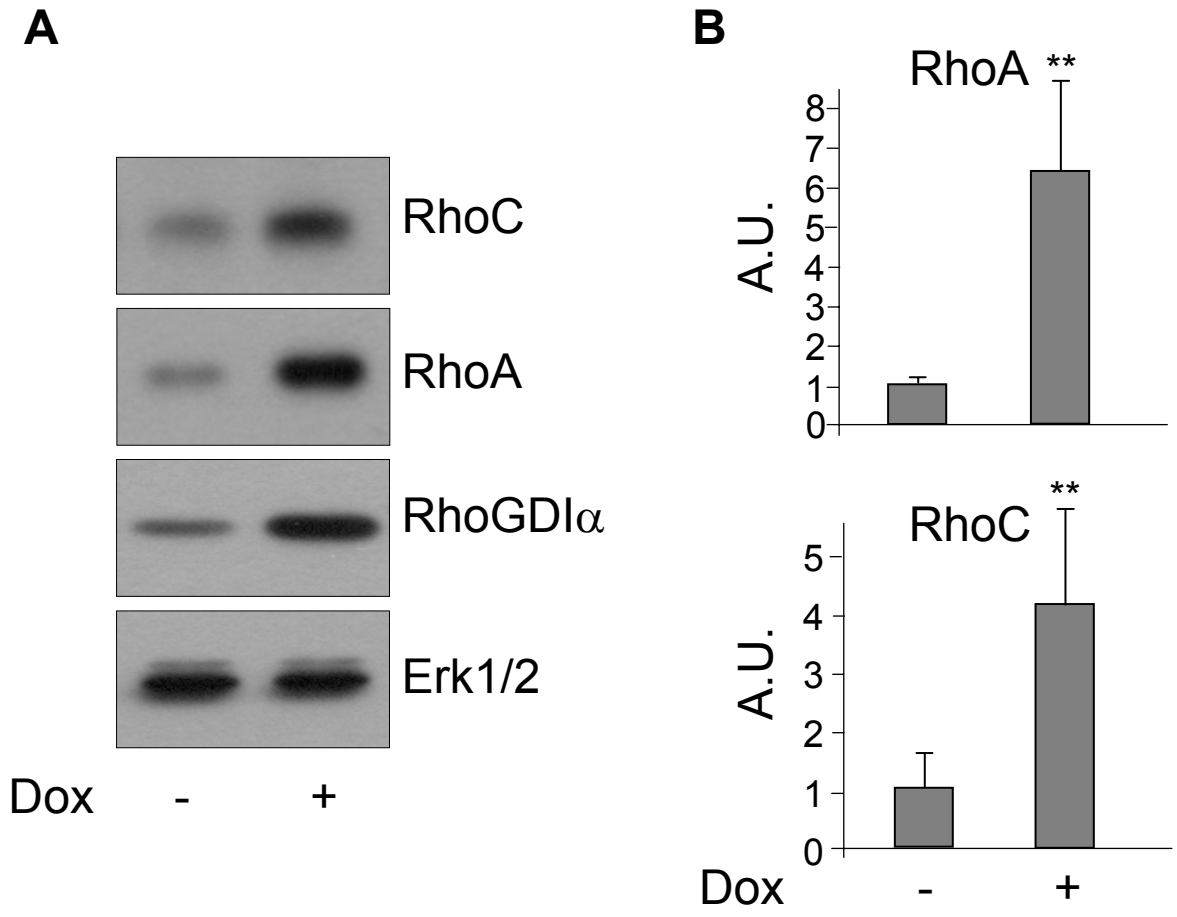
The simultaneous silencing of RhoA and RhoC increased the Rac1 protein level and the interaction between Rac1 and RhoGDIalpha while the activity of Rac1 was decreased. (A-B) PC-3 cells were transfected with 20 or 40nM of an irrelevant siRNA (Scr or Scr[2X]), with 20nM of siRhoA, with 20nM of siRhoC or with 20nM of siRhoA+20nM of siRhoC (siRhoA+C). (A) 48 hours post-transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to RhoA, RhoC, Rac1 and Erk 1/2. The bottom panel illustrates the mean±s.d. of 3 independent experiments. (B) 48 hours post-transfection, cells were lysed and processed for immunoprecipitation with specific antibody to RhoGDIalpha. Before immunoprecipitation, an aliquot of each lysate was denatured in SDS-PAGE loading buffer to analyse the concentration of Rac1 (Input). The immune complexes were analysed by immunoblotting. The IP/input ratio was calculated from densitometric measurements of Rac1 co-immunoprecipitated with RhoGDI normalized to the Rac1 amount present in the lysates (Input) in each transfection condition compared to the Scr condition taken as 1.0. (C) PC-3 cells expressing in an inducible way RhoA were transfected with 20nM of specific control for siRhoA+20nM of specific control for siRhoC (ctrA+C) or with 20nM of siRhoA+20nM of siRhoC (siRhoA+C) and were supplemented (+) or not (-) with doxycycline (100 ng/ml). After transfection, cells were processed for immunoblotting and pull-down experiments. The bottom panel illustrates the mean±s.d. of 3 independent experiments.

Supplemental Figure 7



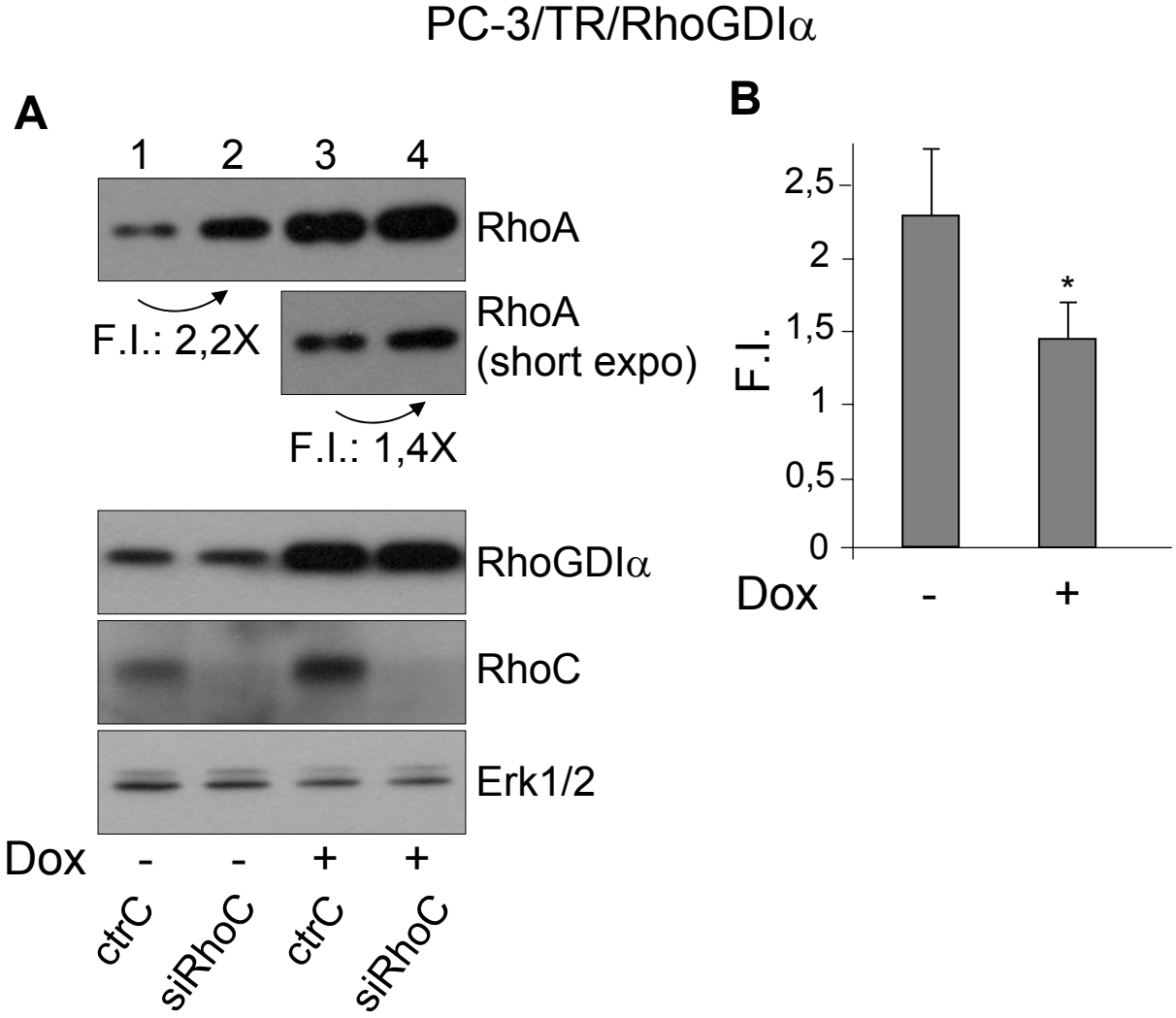
The overexpression of RhoA or RhoC in PC-3 did not modulate the expression or the activity of Rac1 and Cdc42. PC-3 cells overexpressing RhoA (PC-3/TR/RhoA) or RhoC (PC-3/TR/RhoC) in an inducible way were supplemented or not with doxycycline (100 ng/ml). After 48 hours of culture, cells were harvested and processed for Western blot and pull-down experiments. An aliquot of each lysate was denatured in SDS-PAGE loading buffer to analyse the concentration of total Rac1, Cdc42 and Erk1,2 with specific antibodies. Rac1 and Cdc42 activities were determined as the amount of GST-PBD-bound Rac1 (Rac1-GTP) or GST-PBD-bound cdc42 (cdc42-GTP) normalized respectively to total Rac1 or total cdc42 in whole cell lysates. Results are the mean±s.d. of 3 independent experiments.

Supplemental Figure 8



The overexpression of RhoGDIα increased the concentration of RhoA and RhoC. PC-3 clones overexpressing RhoGDIα (PC-3/TR/RhoGDIα) in a doxycycline-dependent way were supplemented or not with doxycycline (100 ng/ml). After 48 hours of culture, cells were lysed and analyzed by immunoblotting with specific antibodies to RhoA, RhoC, RhoGDIalpha and Erk1/2. (A) Representative blots of independent experiments carried out with 4 different clones are shown. (B) The panel illustrates the mean±s.d. of densitometric measurements of RhoA and RhoC in 4 independent experiments expressed in arbitrary units (A.U.). **p<0.01.

Supplemental Figure 9



The overexpression of RhoGDIα repressed the cross-regulation between RhoA and RhoC. PC-3 clones overexpressing RhoGDIalpha (PC-3/TR/RhoGDIalpha) in a doxycycline-dependent way were transfected with 20nM of the indicated siRNA. After overnight transfection, the cells were divided in two wells and supplemented or not with doxycycline (100 ng/ml). After 48 hours of culture, cells were lysed and analyzed by immunoblotting with specific antibodies to RhoA, RhoC, RhoGDIα and Erk1/2. The fold induction (F.I.) is calculated from densitometric measurements of RhoA normalized to Erk1/2 taking the ctrC condition as 1.0 . To avoid artefacts due to over-exposure of x-ray films, the quantifications of RhoA in cells overexpressing RhoGDIα was also carried out by exposing X-ray film 3 time less (short expo). (A) Representative blots of independent experiments carried out with 3 different clones are shown. In control conditions (ctrC), the forced expression of RhoGDIα (+Dox) results in increased levels of RhoA, and to a lesser extent, RhoC (compare lane 1 and 3). Upon treatment with siRhoC, the accumulation of RhoA is lower in cells with forced expression of RhoGDIα (3-4) than in cells expressing only endogenous RhoGDIα (1-2). (B) The panel illustrates the mean±s.d of F.I. calculated from densitometric analysis of independent experiments carried out with 3 different clones of PC-3/TR/RhoGDIα. *$p<0.05$ ANOVA followed by Tukey-Kramer analysis.